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CERTIFICATE

This certificate is issued in support of an application for Patent registration in a country outside New Zealand pursuant to the Patents Act 1953 and the Regulations thereunder.

I hereby certify that annexed is a true copy of the Provisional Specification as filed on 10 August 2000 with an application for Letters Patent number 506287 made by DIATRANZ LIMITED.

Dated 1 February 2001.

Neville Harris
Commissioner of Patents

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506287

NEW ZEALAND

PATENTS ACT, 1953

PROVISIONAL SPECIFICATION

"Preparation of Porcine Islets"

We, DIATRANZ LIMITED, a company duly incorporated under the laws of New Zealand of 19 Laureston Avenue, Papatoetoe, Auckland, New Zealand, do hereby declare this invention to be described in the following statement:

The present invention relates to improvements in and/or relating to the treatment of diabetes using porcine islets conditioned by exposure to GPE.

The present invention relates to the treatment of a mammalian patient suffering from diabetes (including humans) which involves the transplantation into the mammal of viable encapsulated porcine islets capable of producing insulin within its host.

Human Insulin-like Growth Factor I (IgF_1) is a potent mitogenic growth factor that mediates the growth promoting activities of growth hormone postnatally. Both IGF-1 and IGF-2 are expressed in many cell types and may have endocrine, autocrine and paracrine functions. GPE is the amino-teminal tripeptide glycine-proline-glutamate of IGF-1.

Type 1 (insulin-dependent) diabetes mellitus is a common endocrine disorder that results in substantial morbidity and mortality, and leads to considerable financial costs to individual patients and healthcare systems.

Treatment with insulin, while life-saving, often does not provide sufficient control of blood glucose to prevent the feared complications of the disease, which has provided the impetus for intensive research into better methods of sustaining normoglycaemia.

Among the newer treatment strategies that have been proposed, transplantation of pancreatic β islet cells, obtained either from other humans or animals, has received the most attention worldwide. This is because transplantation can restore not only the insulin-secreting unit, but also the precise fine tuning of insulin release in response to multiple neural and humoral signals arising within and beyond the islets of Langerhans.

Human islet cell transplantation is limited by the shortage of human islet tissue. The use of pig islet cells is currently viewed as the most promising alternative since:

(a) the supply of pig cells can be readily expanded by optimising the supply of donor animals;

(b) pig and human insulin have close structural similarities; and

(c) physiological glucose levels in pigs are similar to those in humans.

The rationale for this treatment approach (termed 'xenotransplantation') is that the implanted pig islets have the potential to mimic the normal physiological insulin response in type 1 diabetics such that near-normal blood glucose levels may be achievable without insulin administration or with a reduced requirement for it. As a consequence, long-term diabetes complications may be prevented and patients should experience less hypoglycaemia than they do with the currently recommended 'intensive' insulin regimens.

In one aspect the present invention consists in **viable porcine islets** useful in the treatment of diabetes that have been isolated from a pig (preferably piglets at or near full term gestation, ie; -20 to +10 days full term gestation) and which have been treated with at least GPE as hereinafter defined.

In a further aspect the present invention consists in a **method of isolating viable**

piglets from the pancreas of neonatal piglets which involves a procedure of harvesting the pancreas of piglets at or near full term gestation, and extracting (preferably by an enzyme extraction procedure) pancreatic β islet cells from those pancreas, there being treatment at some stage of such islets (whether once isolated or not) with nicotinamide and GPE (whether such exposure to nicotinamide and GPE is simultaneous or otherwise).

In another aspect the invention consists in **a method for treatment of a mammalian patient** suffering from diabetes which comprises:

(a) extracting pancreatic β islet cells from piglets at or near full term gestation;

(b) treating said islets with nicotinamide,

(c) encapsulating or otherwise confining said islets in a biocompatible material which will allow *in vivo* glucose movement to and insulin movement from the islets, and

(d) injecting or otherwise implanting the encapsulated or otherwise confined islet cells of step (c) so as to transplant into said mammalian patient an effective amount of viable piglet islet cells capable of producing insulin in the patient, and

(e) (optionally) administering nicotinamide to said mammalian patient at least subsequent to transplantation; and

(f) (optionally) administering a casein-free diet to said mammalian patient, the procedure being further characterised in that the islets prior to implantation,

wherein said pancreatic β islet cells at some stage after extraction from the piglets and prior to encapsulation are exposed to GPE.

As used herein "administering" includes self administering.

Preferably said piglets at or near full term gestation from which the pancreatic β islet cells are extracted are at from -20 to +10 days full term gestation.

Preferably said piglets are at from -7 to +10 days full term gestation.

Preferably said exposure to GPE is greater for those cells from piglets furthest from full term gestation but preferably there is exposure to GPE for all cells extracted irrespective of their relationship to full term gestation.

Preferably said encapsulation is with an alginate material (preferably sodium alginate) (whether after pre-coating or not with some other material).

Preferably said alginate (preferably sodium alginate) is in ultra pure form.

Preferably each islet or grouping of islets is entrapped in an *in vivo* insulin and glucose porous biocompatible alginate or alginate like surround.

Preferably such coating prevents, once implanted, direct tissue contact with said islets and/or any pre-coating matrix which itself has the requisite porosity once implanted.

Preferably each encapsulation involves presenting islets and a suitable alginate

solution into a source of compatible cations thereby to entrap the islets in a cation - alginate gel.

Preferably said cation alginate gel is calcium-alginate gel.

Preferably said alginate used in the solution is sodium alginate.

Preferably the islets and sodium alginate solution (preferably 1.6% w/w) is presented as a droplet (eg. through a droplet generating needle) into a bath of suitable cations (eg. gelating cations such as calcium chloride).

Preferably the gel encased islets are coated with a positively charged material and thereafter optionally are provided with an outer coat of a suitable alginate.

Preferably said positive charging material is poly-L-ornithine.

Preferably the gel entrapping the islets within the outer coating is then liquified.

Preferably said liquification is by the addition of sodium citrate.

Preferably said capsules contain a plurality of islet cells (preferably about three) and preferably have a diameter of from about 300 to 400 microns.

After liquification of the alignate entrapping the islets, the "capsules" are washed, and again coated with alignate which neutralizes any residual change on the poly-L-ornithine coating and prevents direct contact of the poly-L-ornithine with tissues when the entire capsule is transplanted.

Preferably the alginate production process has involved the following steps:

Seaweed harvest→Washing→Alginate extraction→Filtration (preferably a 0.2 μm filter→Precipitation→Drying.

Preferably the ultrapure alginate is Kelco LV produced by Monsanto-Kelco, US and has the following specifications

1. Viscosity: 2% - 100-300 cps (Brookfield 25°C, speed 3,60 rpm)
2. pH: 6.4-8.0
3. Protein content <0.5%
4. Filtration: through 0.2 μm
5. Chemical analysis:

Ca: <100 ppm	Mg <40 ppm	Mn: <10 ppm
Cu: <40 ppm	Zn: <40 ppm	Sr: <40 ppm
Fe: <60 ppm	Pb: <50 ppm	As: <100ppb
Hg: <40 ppb	Si: <10 ppm	

6. Endotoxin level - measured by LAL test (at University of Perugia): 39 EU/g [NB. Any level below 100 EU/g in this test is considered endotoxin-free].
7. Molecular weight: 120,000 - 190,000 kD
8. Mannuronic acid (M) content: M fraction (F_m) 61%
9. Guluronic acid (G) content: G fraction (F_G) 39%

Preferably the filtration has been with a multiple filtration process employing positively charged filters that remove any lipopolysaccharide content.

Preferably said extraction involves the use of a trauma protecting agent for the islet cells during the isolation and/or preparation thereof for encapsulation.

Preferably said agent is a trauma protecting agent selected from suitable anaesthetic agents such as, for example, lignocaine.

Preferably the mammalian patient is administered nicotinamide prior to transplantation.

Preferably the casein-free diet is administered to the mammalian patient at least after transplantation.

Preferably a casein-free diet is administered prior to transplantation.

In a further aspect the present invention consists in **encapsulated or otherwise confined pancreatic islets** of a kind useful in a method aforesaid.

In still a further aspect the present invention consists in **a method of porcine β islet cell production and/or method of xenotransplantation thereof in an encapsulated form** when performed by a procedure substantially as hereinbefore described and/or substantially as hereinafter described and/or as shown in Figure 1 of the accompanying drawings.

In still a further aspect the present invention consist in a **xenotransplantable capsule or encapsulated mass** of at least one porcine pancreatic β islet cell comprising at least one viable porcine pancreatic β islet cell enclosed in an *in vivo* glucose porous and insulin porous biocompatible material, said islet(s) having been extracted at -20 to +10 days full term gestation (preferably -7 to +10) and having been exposed to IgF_1.

Preferably said GPE exposure is post isolation and pre-encapsulation.

Preferably said treatment prior to use is as described hereinbefore or in the accompanying drawing.

The present invention also consists in the use of the capsule(s) or mass(es) of the present invention.

The major advantage of porcine islet cell transplantation over human islet cell transplantation is that the islet cell source can be readily expanded, and the biosafety of the cells can be thoroughly explored prior to transplantation. From a practical viewpoint, pancreas removal and islet cell isolation can be performed expeditiously in an ideal environment.

Important considerations relevant to the use of porcine islet cells in transplantation approaches for type 1 diabetes include the following:

- The structural and biological similarities of porcine and human insulin
- The fact that porcine insulin has been used to treat diabetes for several decades

(and has only been replaced by human sequence insulin relatively recently); and

- The similarity of physiological glucose levels in pigs and humans. (Weir & Bonner-Weir 1997). This effectively means that pig islet cells can be expected to react similarly to their human counterparts in maintaining equivalent blood glucose concentrations.

1. ***The nature of the disease causing diabetes:*** successful long-term allotransplantation of human islets can be achieved in over 80% of patients when the disease is caused by non-immune processes. In contrast, even islets obtained from a non-diabetic twin cannot reverse autoimmune diabetes long-term in the diabetic twin member. This emphasises the critical role of autoimmunity in the failure of islet transplantation. This observation has been validated in allotransplantation of rodents with diabetes caused by autoimmunity as compared with diabetes due to pancreatectomy or chemical β cell destruction. No large animal model of autoimmune diabetes exists.

It is possible that the use of islets from different species (xenotransplantation) could avoid autoimmune destruction of transplanted islets, as the immune process of xenotransplant rejection is different to that of allotransplant rejection, but this is entirely hypothetical in humans.

2. ***The viability of the islets:***

The processes by which islets are purified prior to transplantation are traumatic to these highly specialised tissues. Such trauma can induce necrosis or apoptosis – the latter being quite delayed.

Further trauma may result from encapsulation. Processes used by us in both the preparation of islets and their encapsulation have been optimised to ensure minimal damage to the islets. Such procedures have ensured zero warm ischaemia (compared with hours with most human islet preparations), have involved the use of nicotinamide to enhance successful *in vitro* explantation, have involved minimal incubation time with collagenase, have involved swift non-traumatic encapsulation technology, etc. Our preferred preparation preferably uses neonatal (7-day old) islets which is crucial in both limiting islet trauma during purification, and assuring sufficient maturation of the islets for stimulated insulin production to occur.

3. ***Drugs used in the recipient:*** transplantation does not require and avoids the need for cytotoxic agents to suppress the immune system. Such agents are able to enter the alginate microcapsule and cause islet toxicity, as well as causing systemic toxicity. Instead, nicotinamide and a special diet are used (for rationale, see section 1.4 below).

The transplantation procedures of our earlier patent specification have the ability over a period prior to rejection of providing porcine insulin. In this respect, we ourselves conducted clinical trials.

Four type 1 diabetic adolescents received 10,000 free islets/kg bodyweight by intraperitoneal injection. The islets were located from term piglets using the standard collagenase digestion, purification and culture techniques described in section 3.2. All four recipients received oral nicotinamide (1.5 g/day) and a casein-free diet both pre- and post-transplantation. A prompt reduction in insulin requirements, which was not clearly dose-related, was noted in the first week after transplantation. The reduction in insulin dosage range from 21 to 32%, and the response lasted for up to 14 weeks. However, insulin doses subsequently returned to their previous levels.

The most likely reason for the transplant failure in these patients was chronic rejection. However, no adverse effects were noted.

We have now shown alginate-encapsulated porcine islet cell transplants in two human diabetic patients, prolonged functioning of the transplants. The islets were transplanted by intraperitoneal injection, one patient receiving 15,000 IEQ/kg (total 1,300,000 islets) and the other 10,000 IEQ/kg (total 930,000 islets). Both patients were treated pre- and post-transplantation with oral nicotinamide and a soy-based/casein-free diet.

The preferred procedure as shown in Figure 1 was used for the preparation, the encapsulation being as aforesaid. Islet cells of -7 days to +10 days full gestation were used.

The present invention is illustrated by the following examples, but is not to be limited thereby

- porcine islets in culture which were exposed to IGF incorporating the amino terminal glycine-proline-glutamate (hereafter IGF-1(GPE)), increased their insulin response to glucose, by up to a 3-fold increase.

Table 1

	Incubated 24hrs with 0.1ug/ml IGF-1(GPE) after isolation	**CONTROL no IGF-1(GPE)**
Insulin secretion In response to 19.4mM Glucose + 10mM Theophyline After 3 days culture Post isolation	236uU/hr/100IEQ	75.2uU/hr/100IEQ

- A concentration of 0.1ug/ml IGF-1(GPE) in culture is sufficient to produce optimal insulin secretion during glucose challenge. No further benefit was achieved by increasing the concentration of IGF-1(GPE).

	Incubated 24hrs with 0.1ug/ml IGF-1 (GPE)	**Incubated 24hrs with 1.0 ug/ml IGF-1(GPE)**
Insulin secretion In response to 19.4mM Glucose + 10mM Theophyline After 3 days culture Post isolation	58uU/hr/100IEQ	56.8uU/hr/100IEQ

- Variations on the duration of IGF-1(GPE) exposure were tried on the porcine islet cells. However no increased benefit was found on culturing the islets with IGF-1(GPE) beyond a 24hrs period, post isolation.

	Incubated 7 days With 0.1ug/ml IGF-1(GPE)	**Incubated 24 hrs with 0.1ug/ml IGF-1(GPE)**
Insulin secretion In response to 19.4mM Glucose + 10mM Theophyline 7days post isolation	58uU/hr/100IEQ	57.5uU/hr/100IEQ

- This increased insulin production persisted to 14 days post IGF-1(GPE) exposure. Longer duration's are yet to be investigated.

	14 days post IGF-1(GPE) Exposure	**3 days post IGF-1(GPE) Exposure**

Insulin secretion In response to 19.4mM Glucose + 10mM Theophyline	1.3-fold increase Compared to control	1.5-fold increase Compared to control

- Withdrawal of Nicotinamide from the culture media eliminated the benefit of IGF-1(GPE) on islet insulin production.

	Incubated 3 days With 0.1ug/ml IGF-1(GPE) Without Nicotinamide	**Incubated 3 days With culture Media Without Nicotinamide**
Insulin secretion In response to 19.4mM Glucose + 10mM Theophyline After 3 days culture Post isolation	47.6uU/hr/100IEQ	55.9uU/hr/100IEQ

- A concentration of 0.1ug/ml IGF-2 during culturing appeared to increase insulin production of porcine islet cells, after an initial exposure of 24 hrs. However, this increase was transient to 3 days post exposure.

	Incubated 24hrs With 0.1ug/ml IGF-2 day 1.	**Control**
Insulin secretion In response to 19.4mM Glucose + 10mM Theophyline After 3 days culture Post isolation	105.8/100IEQ	75.2r/100IEQ

	Incubated 24hrs With 0.1ug/ml IGF-2 day 1.	**Control**
Insulin secretion In response to 19.4mM Glucose + 10mM Theophyline After 7 days culture Post isolation	32uU/hr/100IEQ	39.8 uU/hr/100IEQ

- Prolonged exposure to IGF-2 beyond 24hrs, failed to increase the insulin production of the islet cells in response to glucose.

	Incubated 24hrs With 0.1ug/ml IGF-2 day 1.	**Control**
Insulin secretion In response to 19.4mM Glucose + 10mM Theophyline After 3 days culture Post isolation	105.8/100IEQ	75.2r/100IEQ

	Incubated 7 days With 0.1ug/ml IGF-2	**Control**
Insulin secretion In response to 19.4mM Glucose + 10mM Theophyline After 7 days culture Post isolation	38.4uU/hr/100IEQ	39.8uU/hr/100IEQ

The effect of Lidocaine when used during porcine pancreatic digestion, on islet yield and viability.

Lidocaine is a membrane stabiliser and phospholipase A2 inhibitor. When used at a 1mM concentration during Collagenase digestion of 7d old porcine pancreas, a 2-fold increase in islet yield is produced.

Islet endocrine function was assessed after 3 days in culture via static glucose stimulation. Islets isolated with Lidocaine during digestion produced a 3-fold increase in insulin secretion in response to glucose challenge.

	Collagenase alone	Collagenase + 1mM Lidocaine
Average islet yield	40,960 IEQ/g	88,183 IEQ/g

	Collagenase alone	Collagenase + 1mM Lidocaine
Insulin secretion in response to 19.4mM Glucose + 10mM Theophyline After 3 days culture Post isolation	46.4 uU/hr/100IEQ	163.8 uU/hr/100IEQ

Conclusion: The use of Lidocaine during pancreatic digestion increases the insulin production/g of pancreas by 6-fold.

DATED THIS 10th DAY OF August 2000
AJ PARK
PER J. Filary
AGENTS FOR THE APPLICANT

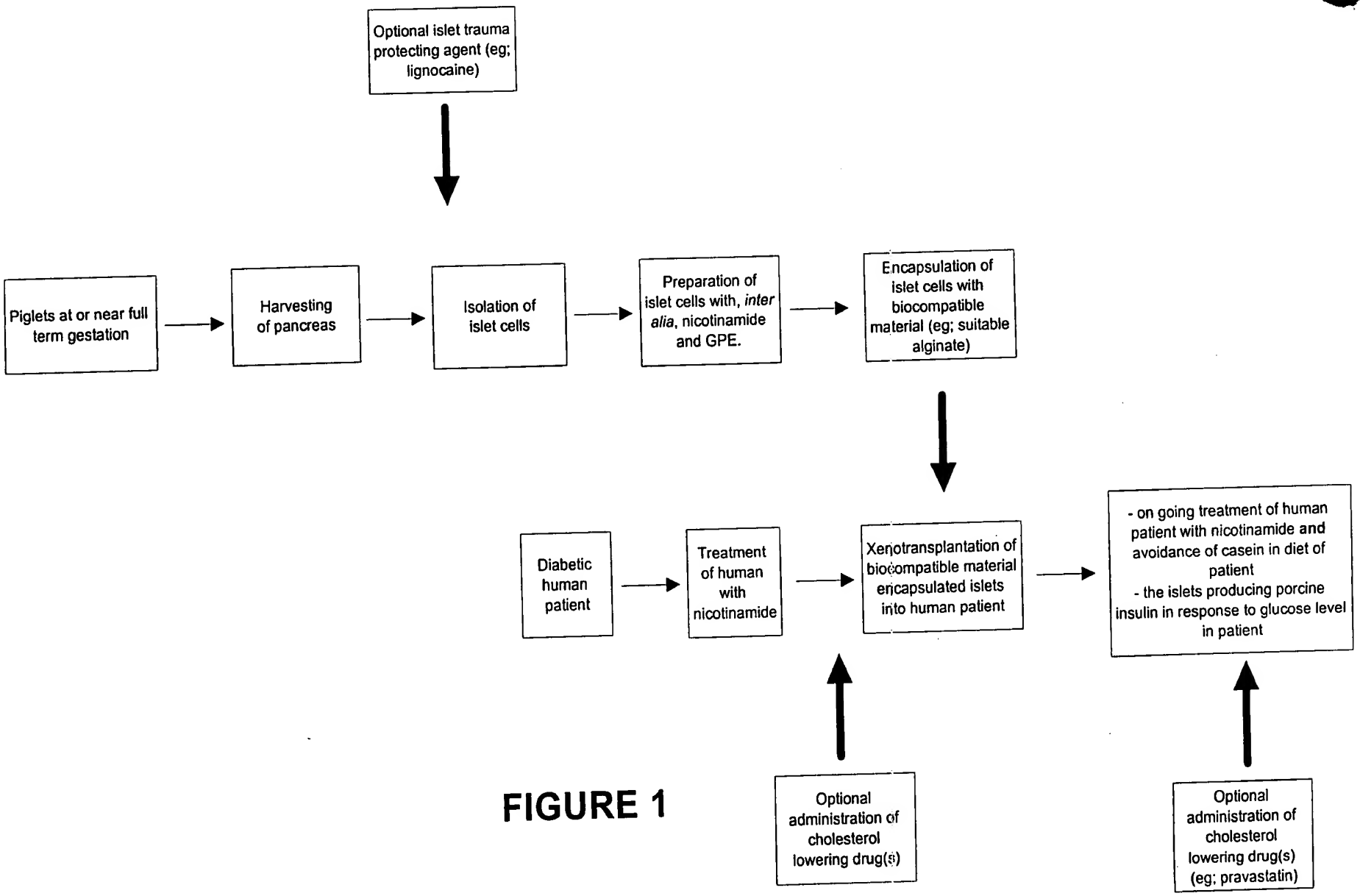

FIGURE 1